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Exhibit A

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Bachovchin

Serial No: 09/601,432

Filed: January 5, 2001

For: Method of Regulating Glucose
Metabolism, And Reagents Related
Thereeto

Attorney Docket No. TUU-P02-006

Art Unit: 1654

Examiner: J. Russel

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Declaration Under 37 CFR 1.131 of Daniel Drucker

Sir:

I, Daniel Drucker of Toronto, Canada, hereby declare as follows:

1. I am a co-inventor of the above-mentioned application which teaches and claims uses of dipeptidylpeptidase IV inhibitors for treatment of Type II diabetes, modifying GLP-1 metabolism, and modifying glucose metabolism, among other things.
2. The invention as described and claimed in the above-identified application was conceived prior to the publication of Balkan et al., Diabetologia, Suppl 40, A131 Abstract, which published in June of 1997.
3. In support of this, I attach as Exhibit A the text of an e-mail from me to coinventor Andrew Plaut memorializing our decision to administer Pro(boro)Pro in an oral glucose tolerance test (OGTT) in mice to confirm its effectiveness for treating Type II diabetes, modifying GLP-1 metabolism, or modifying glucose metabolism. See Exhibit B for the structure of Pro(boro)Pro. This compound is a potent dipeptidylpeptidase IV inhibitor. The OGTT in this case measures the efficacy of Pro(boro)Pro in reducing blood glucose levels when administered to mice that had ingested a measured amount of glucose after a period of fasting. I sent the e-mail of Exhibit A prior to June 1997.
4. After sending the e-mail of Exhibit A, the OGTT experiments were planned to be carried out using mice having normally functioning GLP-1 processes (GLP-1 +/+ mice) and GLP-1 knockout mice (GLP-1 -/- mice). (See Exhibit C) During the four months after sending the e-mail of Exhibit A, we received a batch of Pro(boro)Pro from co-inventor Dr. Andrew Plaut and ordered the GLP-1 +/+ mice, the GLP-1 -/- mice, and other materials required to carry out the experiment. The OGTT experiments were commenced within four months of having sent the e-mail of Exhibit A, and results were obtained within two months after that. The results showed that Pro(boro)Pro improves glucose tolerance in both mice models. (See Exhibit D)

5. Type II diabetes is characterized by glucose intolerance. The mice used in our experiments are reasonable models of glucose intolerance.

6. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title XVIII of the United States Code and that willful false statements may jeopardize the validity of this Application for Patent or any patent issuing thereon.

Daniel Drucker

Dated: Apr. 1 12 4


Signature: 

Exhibit A

From: IN%"d.drucker@utoronto.ca" 12-MAY-1997
Hi Alfrew,

I may have met a very distant cousin of yours in Baltimore (Allergy and Immunology, now at the NIH?) this weekend.

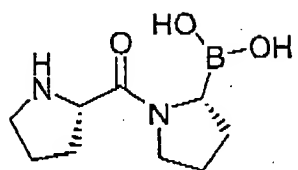
On perusing the papers you sent me, the best experiments would be those we could carry out in rats. This is because a) rats have higher levels of DP-IV than mice and b) we could get more blood from the rats for HPLC/RIA experiments to show the precise molecular forms of peptides in the presence and absence of inhibitors. Any data on the use of the inhibitors in rats from your group? I am extrapolating from the []s of inhibitor used in mice as follows, so correct me if I make a mistake: The [] of inhibitor that is reasonably effective is 1 ug/kg sc twice a day.

If we do rats, then this would be ~250 ug twice a day, x 10 rats for example, x 10 days (for the bowel growth/GLP-2 effects). Do you have these amounts of inhibitor available? If so, we could try and get these studies done in the next few months. We could also do some more acute effects with OGTT and GLP-1 that would be short-term studies that wouldn't require much inhibitor. Let me know if the amount of material required is a problem. DD

*****Daniel

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Toronto, Ontario Canada M5G 2C4

Exhibit B



Pro(boro)Pro

dissolve at pH 2
+ store at 4°C or freeze

Pro(boro)Pro Experiments

Previous Experiments

- Involvement of DPIV in an *in vivo* immune response AND DPIV activity in serum and on lymphocytes of MRL/Mp-*lpr/lpr* mice correlates with disease onset (both Kubota et al.)

• Pro(boro)Pro conc. (ug): 20 40 80 160*
% DPIV activity in plasma: 9.6% 6.7% 3.8% 1.9%
[1 hour following sub. cu. injection (BALB/c mice)]

*No toxicity observed at high doses.

- Dissolve Pro(boro)Pro in saline and filter sterilize.

- Repeated injections of 10 ug Pro(boro)Pro at 12 h intervals maintained *in vivo* DPIV activity at less than 30% of the normal level. (Measured two hours after the fourth injection of 10 ug Pro(boro)Pro at 12h intervals).

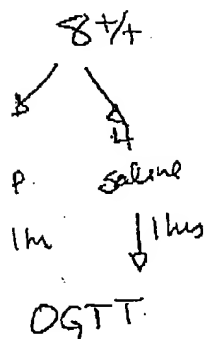
- Assay for DPIV: 10 ul plasma incubated with fluorogenic substrate Ala-Pro-AFC (Enzyme Systems Products, Dublin, CA) in cuvette of a Luminescence Spectrometer.

- Pro(boro)Pro $t_{1/2} = 1.5h$

- Approximately 50% of normal DPIV activity is observed 23h following the last injection.

★ Experiment 1 (Week of Sept. 15)

- 8 male +/- mice, 6-8 weeks old
- 4 receive 160 ug subcutaneous Pro(boro)Pro/saline injection, 1h prior to OGTT
- 4 receive subcutaneous saline injection 1h prior to OGTT.
- Obtain plasma sample at between 20-30 min time point of OGTT to measure DPIV activity and/or plasma insulin levels. GLP-1 levels could also be measured by HPLC or RIA if total bloods were obtained by cardiac puncture following the 120 minute time point (assuming the RIA Ab is able to distinguish between active 7-36 and inactive 9-36 GLP-1)
- Expected Results: Should observe decreased blood glucose excursion in mice receiving Pro(boro)Pro injection due to increased GLP-1/ GIP insulinotropic action. Plasma insulin levels are expected to be increased in mice receiving Pro(boro)Pro, DPIV plasma activity should be drastically reduced, and plasma concentrations of active GLP-1 should be elevated in mice receiving Pro(boro)Pro.
- If glucose tolerance is not significantly improved in mice receiving a single dose of Pro(boro)Pro, administer four consecutive doses of Pro(boro)Pro at 12h intervals, and commence an OGTT 1h following the last injection.



probably won't see much of an effect when only endogenous GLP-1 is present.

Experiment 2

3 -/- mice

- Repeat above experiment with GLP-1R -/- mice. Expect glucose tolerance to be improved in -/- receiving Pro(boro)Pro due to increased GIP action, although not as dramatically as +/+ because GLP-1 signaling is absent. Although there is evidence that GIP sensitivity is enhanced in the -/- mice, apparently in an effort to compensate for the absence of GLP-1, the GLP-1R -/- mice are still glucose intolerant, suggesting that increased GIP sensitivity is insufficient to maintain normal glucose control in these mice. Thus, further increases in GIP action in the -/- mice treated with DPIV-inhibitor is not expected to decrease glucose excursion as dramatically as in +/+ mice treated with Pro(boro)Pro.

Experiment 3

8 +/+ mice

GLP-1
+
PBP

- 8 +/+ male mice
- 4 receive both Pro(boro)Pro (1h prior to OGTT) and GLP-1 (5 min prior to OGTT) injections
- 4 receive a saline injection 1h prior to the OGTT, and a GLP-1 injection 5 minutes prior to OGTT.
- Expected result: Pro(boro)Pro coadministered with GLP-1 should enhance the effectiveness of GLP-1 in suppressing glucose excursion following oral glucose administration. This supports the idea that an analogue of GLP-1 resistant to degradation by DPIV would be more effective in the treatment of NIDDM.

10ug dose - use min. dose to see effect

- ask hawthorne where Pro(boro)pro is stored.

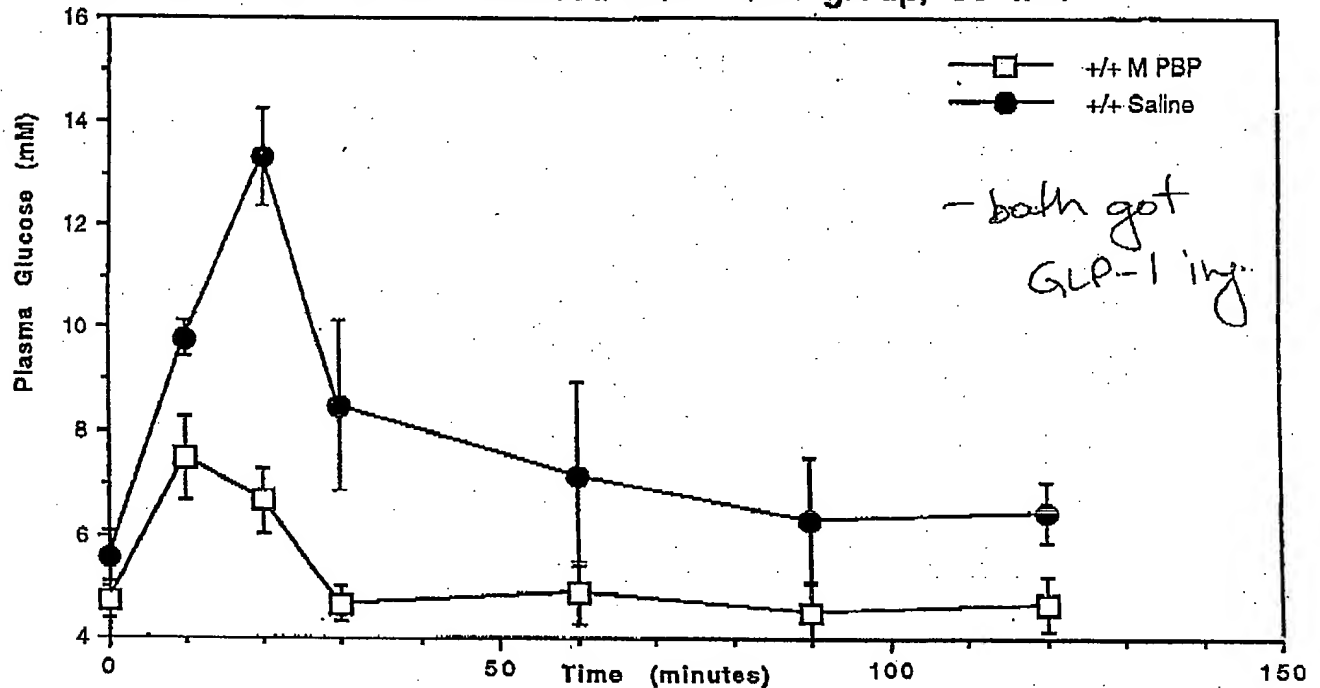
+/+

FIRST - injected PBP 1 hr. prior
to OGTT, then GLP-1 5
mins. prior to OGTT
- gave GLP-1 because we
didn't think we'd see effect
w/ endogenous GLP-1 only

PBP-1

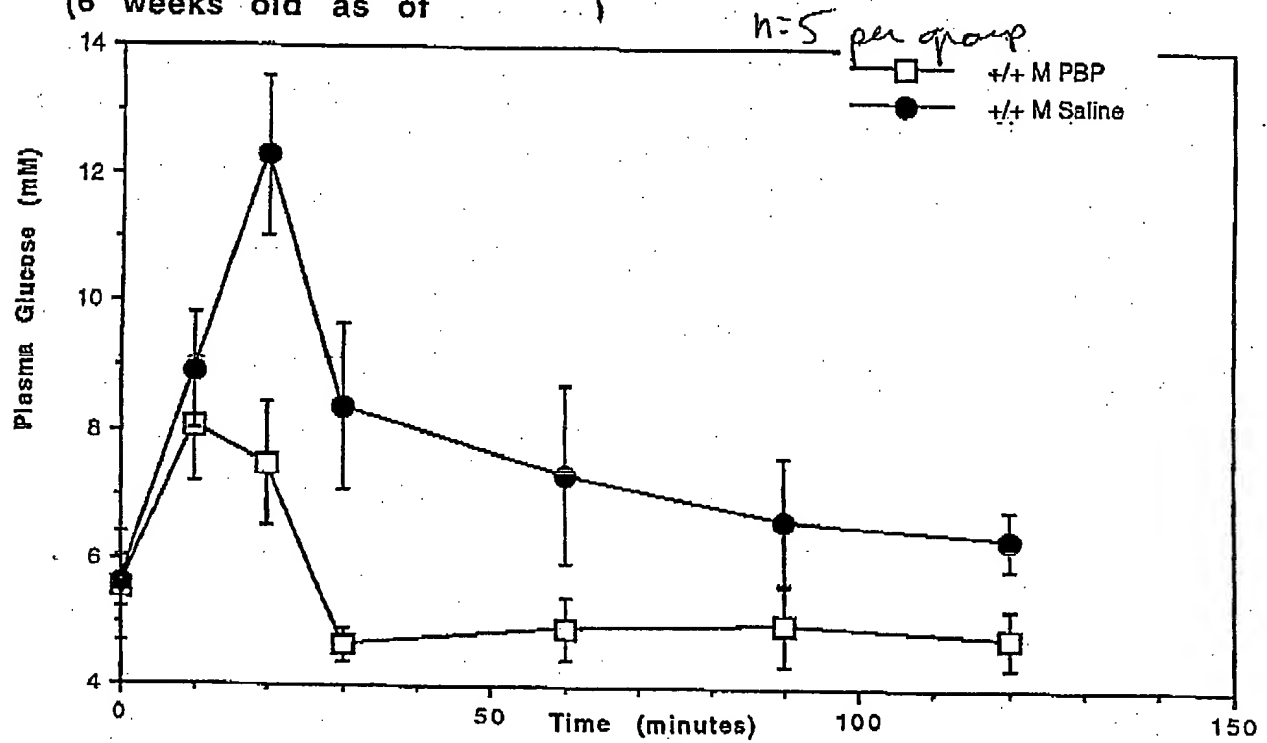
+/+ males, receiving either saline or 150ug Pro(boro)pro 1 hr prior
to oral glucose challenge AND 8 ug GLP-1 5 mins prior to glucose load
(6 weeks old as of)

*Data from one mouse removed from each group, so n=4

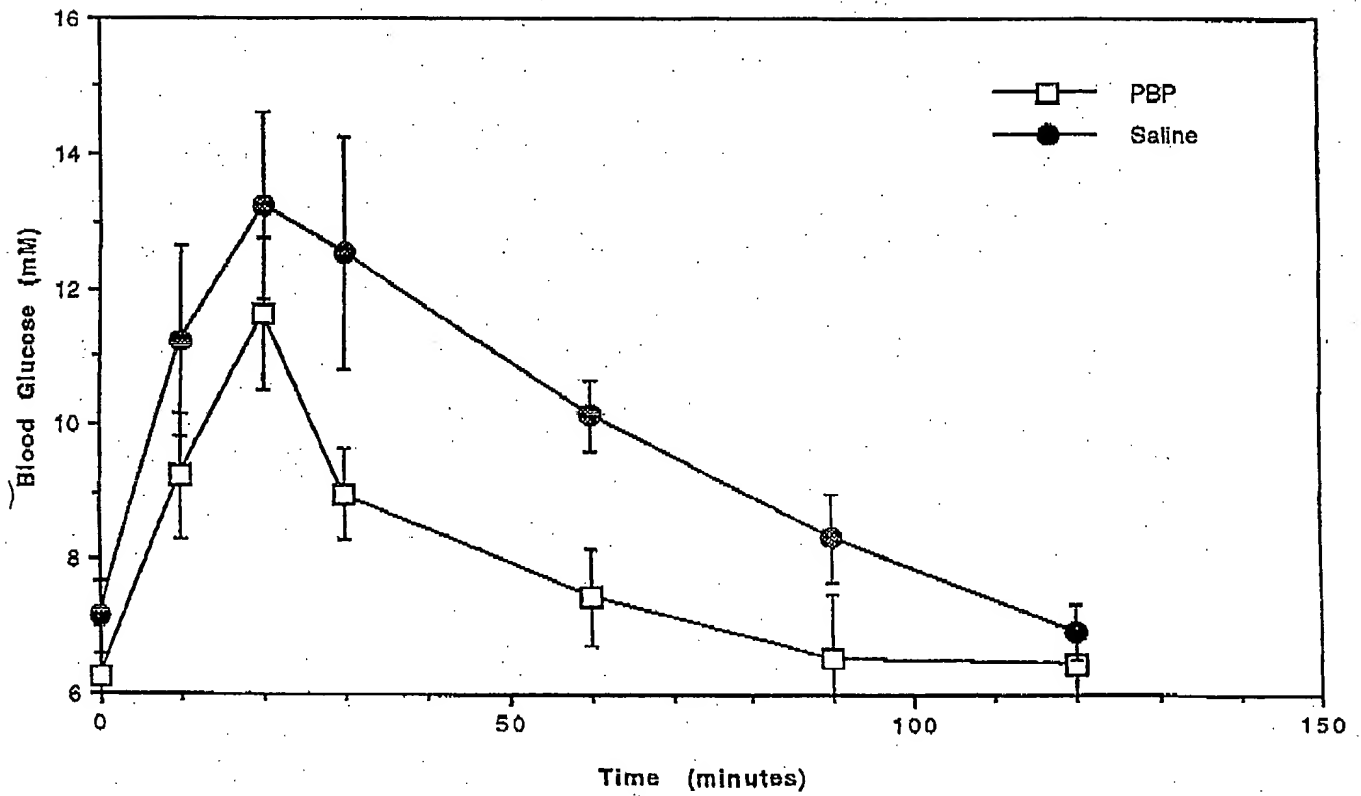


PBP-1

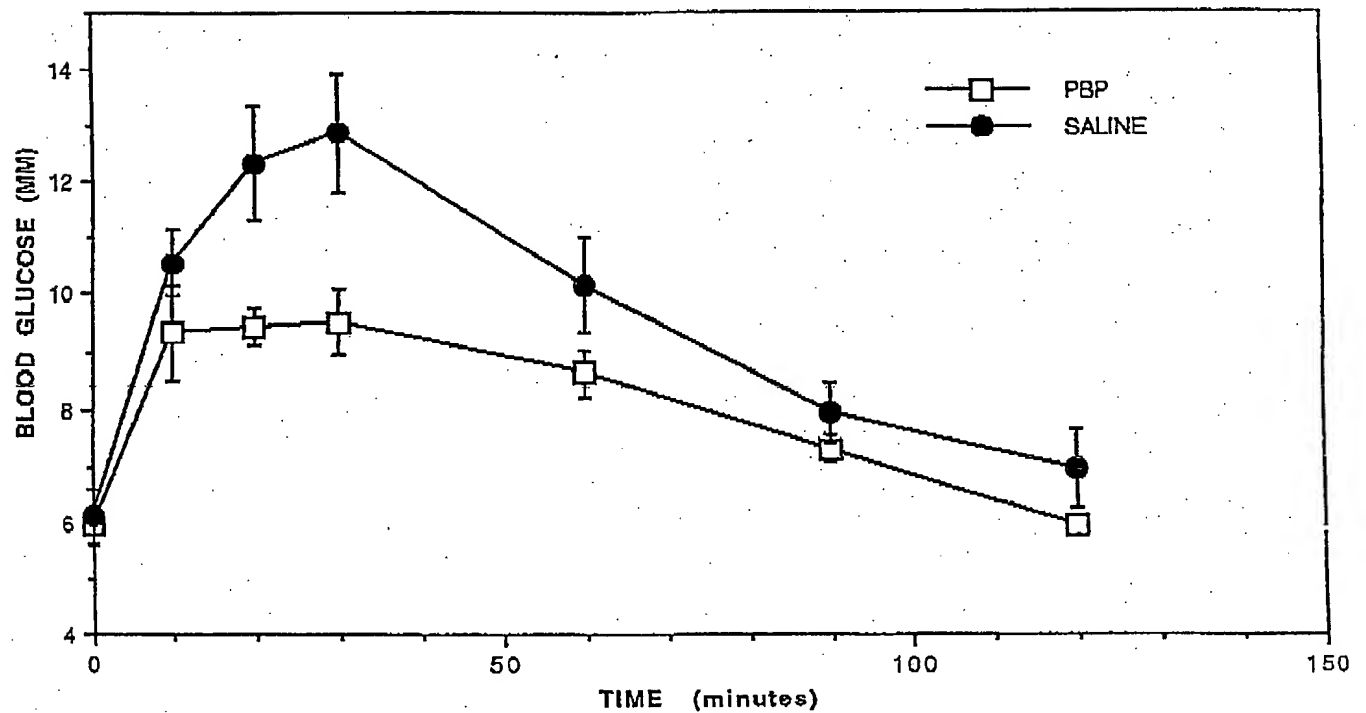
+/+ males, receiving either saline or 150ug Pro(boro)pro 1 hr prior to oral glucose challenge AND 8 ug GLP-1 5 mins prior to glucose load (6 weeks old as of)



PBP-2 \pm Males (6 weeks of age as of) injected with either 150ug Pro(boro)pro or saline 1 hour prior to an OGTT



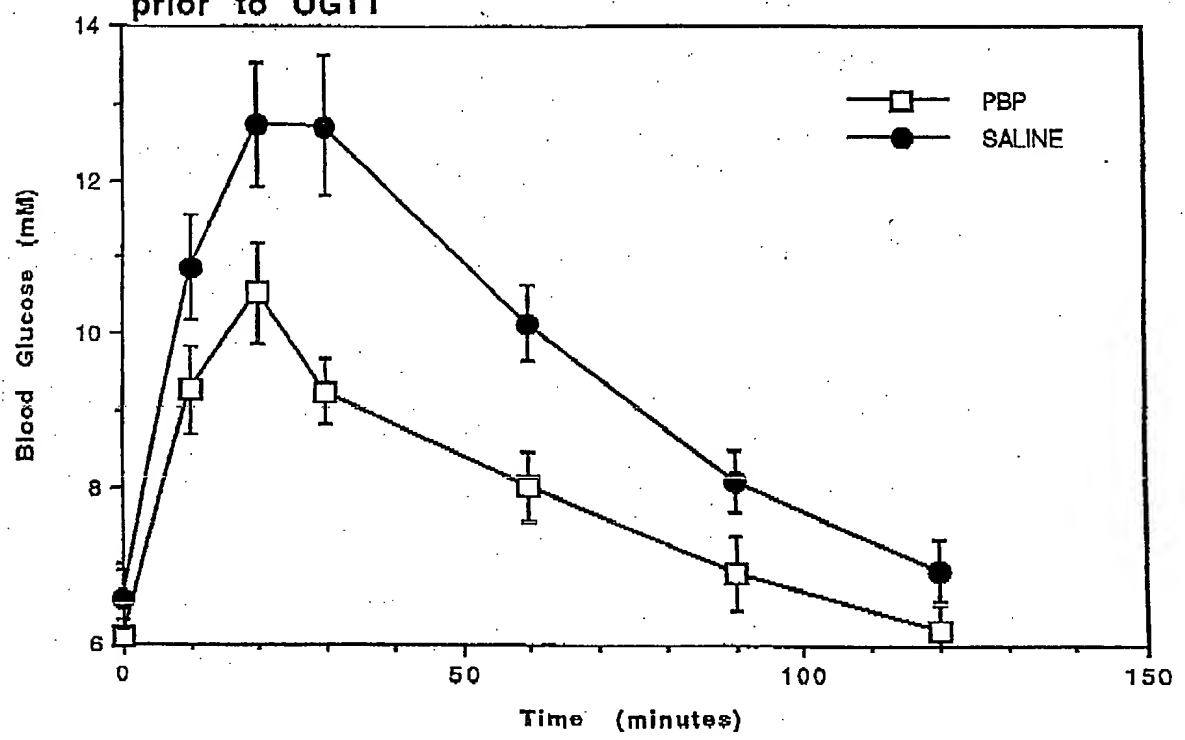
PbP-2 Repeat ^{xx} Male mice (6 weeks old as of) were injected with either 150ug Pro(boror)pro OR saline 1 hr prior to OGTT



Then - just PBP
or saline

+/+

PBP-2 Combined Data ~~x/x~~ Male mice (6 weeks old as of . . .)
were injected with either 150ug Pro(boro)pro OR saline 1 hr
prior to OGTT



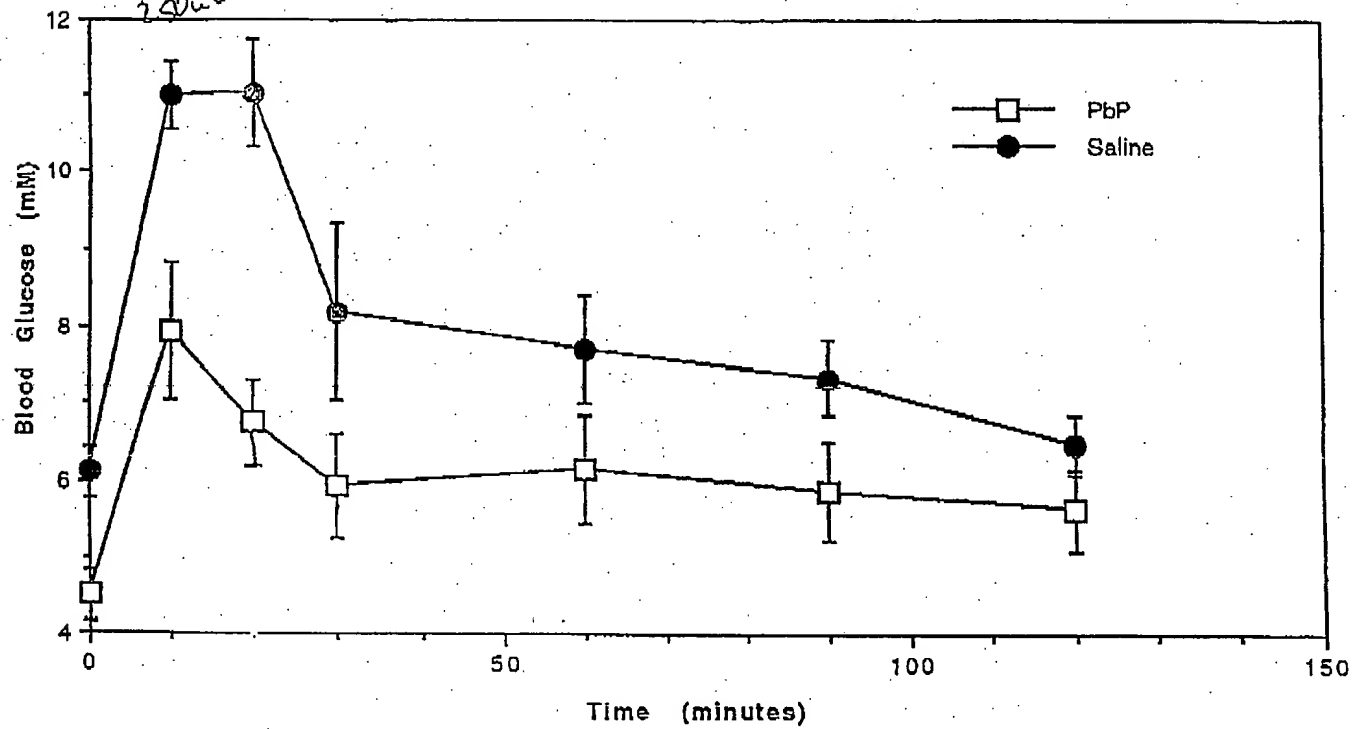
+/+ ♂

250ul Saline + 250ul PBP

IP injection

PBP-3
saline twice daily as of

+/+ Females injected with 150ug Pro(boro)pro OR
(9 injections) 7-8 weeks old as of



Metabolism of glucagon by dipeptidyl peptidase IV (CD26)

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Abstract

Glucagon is a 29-amino acid polypeptide released from pancreatic islet α -cells that acts to maintain euglycemia by stimulating hepatic glycogenolysis and gluconeogenesis. Despite its importance, there remains controversy about the mechanisms responsible for glucagon clearance in the body. In the current study, enzymatic metabolism of glucagon was assessed using sensitive mass spectrometric techniques to identify the molecular products. Incubation of glucagon with purified porcine dipeptidyl peptidase IV (DP IV) yielded sequential production of glucagon_{3–29} and glucagon_{5–29}. In human serum, degradation to glucagon_{3–29} was rapidly followed by N-terminal cyclization of glucagon, preventing further DP IV-mediated hydrolysis. Bioassay of glucagon, following incubation with purified DP IV or normal rat serum demonstrated a significant loss of hyperglycemic activity, while a similar incubation in DP IV-deficient rat serum did not show any loss of glucagon bioactivity. Degradation, monitored by mass spectrometry and bioassay, was blocked by the specific DP IV inhibitor, isoleucyl thiazolidine. These results identify DP IV as a primary enzyme involved in the degradation and inactivation of glucagon. These findings have important implications for the determination of glucagon levels in human plasma. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Rodent; Peptide inactivation; Incretins; Glucose; GIP; GLP-1; MALDI-TOF mass spectrometry

1. Introduction

Glucose homeostasis is maintained in large part by a balance between the anabolic actions of insulin and the catabolic actions of the glucose counter-regulatory hormone, glucagon. Altered secretion and responsiveness of glucagon is a hallmark of diabetes mellitus: hyperglucagonemia is observed in types 1 and 2 diabetes, and there is a diminished glucagon response to hypoglycemia in type 1 diabetes [1].

Glucagon shares sequence identity with the gut hor-

mones glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1). Together, GIP and GLP-1 comprise the incretin component of the entero-insular axis and account for approximately 50% of nutrient-stimulated insulin secretion [2,3]. In recent years, it has been demonstrated by ourselves and others that the circulating serine protease dipeptidyl peptidase IV (DP IV; EC 3.4.14.5; CD26) rapidly cleaves the N-terminal dipeptides from these incretins [4–12] both in vitro and in vivo, yielding truncated, biologically inactive GIP_{3–42} and GLP-1_{9–36amide}. The physiological role played by DP IV in the regulation of incretin activity, and thus in the regulation of blood glucose, was recently established by studies showing enhanced insulin secretion and improved glucose tolerance resulting from the administration of specific DP IV inhibitors [8,10]. Rats lacking the enzyme DP IV were

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shown by us to have normal glucose tolerance, despite having longer acting incretins, leading to the hypothesis of compensatory mechanisms, possibly involving glucagon, that were activated to prevent hypoglycemia [9].

The circulating half-life of glucagon has been reported to be in the range of 5 to 6 min [13,14]. The literature regarding degradation of glucagon by the liver is controversial. It has been reported that hepatic metabolism of glucagon is mediated by the cytosolic enzyme dipeptidyl peptidase I (DP I; EC 3.4.14.1; cathepsin C) [15–17]. However, in reviewing the contribution of the liver to glucagon degradation, Holst concluded that in dogs, as well as humans, there is low hepatic extraction of glucagon [18]. This conclusion is supported by findings that glucagon was not degraded by passage through the perfused rat liver [19]. Consensus exists that the kidney plays a major role in the metabolic clearance and degradation of glucagon [18]. Studies by several groups [20–22] indicated that after glomerular filtration in the kidney, glucagon is hydrolyzed by brush border enzymes in the proximal tubule. Relevant to this finding and the current study is the presence of high concentrations of DP IV in the renal tubular brush border [23,24].

The enzyme DP IV is substrate-specific, preferentially cleaving dipeptides from the N-terminus of peptides with a proline or alanine in the penultimate (P_1) position [25]. Recently, in a study of DP IV utilizing low-molecular-weight peptide substrates, the substrate specificity was extended to include peptides containing P_1 Hyp, Ser, Gly, Val, or Leu residues [25–27]. Thus glucagon, with a P_1 serine, became a potential candidate for DP IV-mediated degradation.

The inclusion of glucagon as a potential DP IV substrate, and the absolute requirement for an intact N-terminus of glucagon for biological activity, therefore provided the rationale for studying glucagon metabolism by DP IV [28,29]. In the current study, products of *in vitro* glucagon hydrolysis were characterized using matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI–TOF MS). *In vivo* studies demonstrated that N-terminal truncation of the peptide by DP IV renders it biologically inactive. In a parallel study, we have shown that N-terminally modified glucagon analogs (truncated, cyclized and/or P_1/P_1 substituted) have reduced binding affinity and efficacy, when tested on cells transfected with the human glucagon receptor [30]. These findings are consistent with a role of DP IV in glucagon degradation.

2. Materials and methods

2.1. Peptide synthesis and purification

Glucagon and synthetic fragments were synthesized using an automated peptide synthesizer SYMPHONY (RAININ), applying an Fmoc-protection protocol. All

reagents for peptide synthesis were analytical grade and obtained from Novabiochem (Schwalbach, Germany), Roth (Karlsruhe, Germany) or J.T. Baker (Griesheim, Germany). Products were purified by HPLC, and MALDI–TOF mass spectrometry was used to confirm peptide identity and purity. Synthetic peptides used in this study are described in more detail elsewhere [30].

2.2. DP IV and inhibitors

Dipeptidyl peptidase IV from pig kidney was purified according to the method described by Wolf et al. [31]. The specific activity was measured spectrophotometrically ($\lambda = 390$ nm) using Gly-Pro-4-nitroanilide as a chromogenic substrate [7,8,12]. Activity of DP IV used in these studies was 31 units/mg protein; one unit of DP IV activity is defined as the rate of appearance ($\mu\text{M}/\text{min}$) of yellow product, 4-nitroaniline, from 400 μM substrate in HEPES buffer (40 mM, pH 7.6, 30°C). Human serum was pooled from healthy subjects, and diluted to 20% in Tris buffer (0.1 mM, pH 7.6) for use in experiments. The highly specific, reversible, competitive transition-state analogue inhibitor of DP IV, isoleucyl thiazolidine (Ile-Thia), synthesized in our laboratory, has been previously described in the literature [8,25]. Generally, analysis of degradation was measured qualitatively and quantitatively using capillary zone electrophoresis or MALDI–TOF MS. Glucagon (2.5 μM) was incubated in either 2.5 mU/ml of purified porcine DP IV or 20% human serum in Tricine buffer with or without Ile-Thia (20 μM) as previously described [7]. Samples were removed at various time points and analyzed.

2.3. Capillary zone electrophoresis (CE)

CE analysis of DP IV-mediated glucagon degradation was carried out on a P/ACE 2050 system (Beckman Coulter GmbH, München, Germany) using a fused-silica capillary (diameter: 50 μm , effective length: 20 cm). Samples were injected with positive pressure for 8 s. A constant voltage (14 kV) was used to separate peptides, using a sodium phosphate buffer (0.1 M, pH 2.5). Absorption of the peptides was monitored at a wavelength of 200 nm. Calculations of enzyme kinetics were performed according to established methods [7].

2.4. Matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI–TOF MS)

MALDI–TOF MS analysis of DP IV-catalyzed peptide proteolysis using a Hewlett–Packard G2025 mass spectrometer has been previously documented [7]. Briefly, at various time points, a 1:1 mixture of analyte and matrix solution (2',6'-dihydroxyacetophenone with diammonium hydrogen citrate) were combined and 1 μl was transferred to the sample tip. The analyte/matrix complex was evapo-

rated immediately in a vacuum chamber (sample preparation accessory HP G2024A), in order to obtain a homogeneous crystallization. Each mass spectrum represents the cumulative m/z signal for 250 single laser shots (laser power 1.0–2.5 μJ).

2.5. Bioassay

In vivo testing of glucagon and N-terminally truncated fragments was carried out using a bioassay monitoring blood glucose concentration. Degradation of glucagon by purified pig kidney DP IV, normal Wistar or Fischer 344 rat plasma, or DP IV negative Fischer 344 rat plasma, with or without DP IV inhibitor (Ile-Thia, 50 μM) was assessed. Degradation of glucagon by purified DP IV was assessed by incubation with 0.31 units of DP IV in 1.0 ml of phosphate-buffered saline (PBS, pH 7.4) at 37°C for 3.25 h, followed by subcutaneous (SC) injection into conscious unrestrained male Wistar rats (225–275 g). Similarly, glucagon was incubated with normal male (Wistar or Fischer) rat serum or DP IV negative rat serum (1.0 ml) under the same conditions (37°C, 3.25 h), prior to SC injection. Concurrent experiments using 50 (micro)M Ile-Thia under the same conditions were assayed by IV injection into anesthetized male Wistar rats (65 mg/kg sodium pentobarbital; Somnotol, MTC Pharmaceuticals, Cambridge, Ontario, Canada). DP IV negative rats were obtained from a colony maintained in the Department of Physiology, UBC, Vancouver, Canada [9]. Control vehicle (saline or plasma) injections were also given in all experimental protocols. Bioactivity of synthetic N-terminally truncated peptides dissolved in saline was tested using analogue doses at ten-fold higher concentration than for native glucagon (doses: 7.1 nmol/kg glucagon_{1–29}, and 71 nmol/kg for glucagon_{3–29}, pyroglutamyl-glucagon_{3–29} {[pGlu³]glucagon_{3–29}} and glucagon_{5–29}). Whole blood glucose concentration was measured using tail bleeds and a SureStep[®] glucose analyser (LifeScan Canada, Burnaby, B.C., Canada). Animal work was performed in accordance with the guidelines set out by Principles of Animal Laboratory Care (NIH publication No. 85-23, revised 1985).

2.6. Data analysis

Data are presented as mean \pm standard error of the mean (S.E.M.), with the number of experiments shown in the figure legends. Statistical significance was set at the 5% level, and assessed using analysis of variance (ANOVA) and Dunnett's Multiple Comparison Test or the Newmann–Keuls Test as post hoc tests of significance where appropriate. Data analysis was done using the Prism software package (GRAPHPAD, San Diego, CA, USA). Blood glucose data are presented as fold-basal activity.

3. Results

3.1. DP IV and serum degradation products of glucagon

Molecular species resulting from incubation of glucagon_{1–29} (3483.8 Da) in purified porcine DP IV were identified by MALDI–TOF mass spectrometry. DP IV-catalyzed loss of the N-terminal dipeptide of glucagon (His¹-Ser²) was indicated by the appearance of glucagon_{3–29} (3259.6 Da) in the analyte (Fig. 1). A second degradation product corresponding to glucagon_{5–29} (3074.6 Da) appeared over a longer time course indicating subsequent N-terminal cleavage of Gln³-Gly⁴ from glucagon_{3–29}. Both degradation steps were completely blocked by the addition of 20 μM isoleucyl thiazolidine (Ile-Thia), a non-hydrolyzable, reversible DP IV-specific inhibitor (data not shown).

Incubation of glucagon in 20% human serum showed a single step degradation to a single molecular species with the molecular weight of 3242.5 Da (Fig. 2A), inclusion of 20 μM Ile-Thia in the incubation prevented formation of this product (Fig. 2B) identifying DP IV as the protease responsible. This product was 17 Da less than the mass of

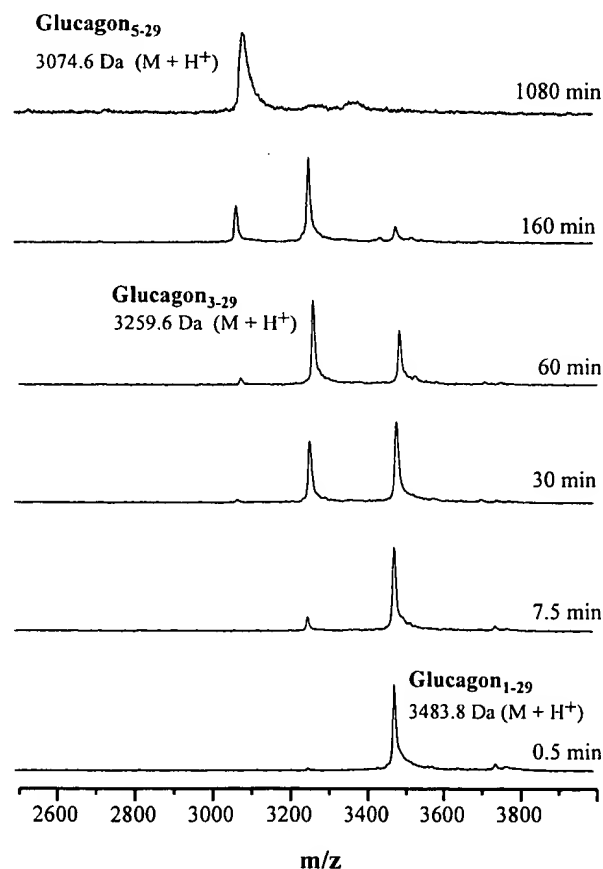


Fig. 1. Degradation of glucagon by purified porcine DP IV monitored by MALDI–TOF mass spectrometry. Refer to Section 2 for specific methods.

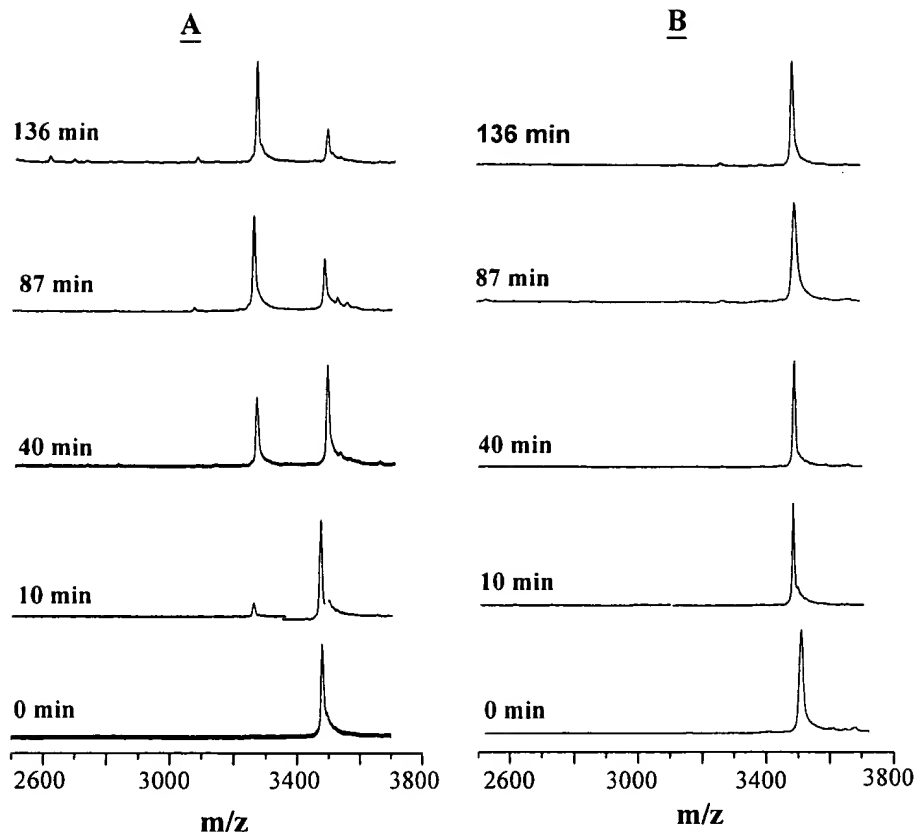


Fig. 2. Degradation of glucagon by 20% human serum monitored by MALDI-TOF mass spectrometry in the absence (A) and presence (B) of the DP IV inhibitor, Ile-Thia. Refer to Section 2 for specific methods.

the expected product, glucagon₃₋₂₉ (3256.7 Da); comparison with synthetic glucagon₃₋₂₉ by MALDI-TOF MS revealed two distinct molecular species (Fig. 3). Incubation of glucagon₃₋₂₉ in 20% human serum for 2 h also gave rise to this slightly smaller molecular species (Fig. 4). Glucagon₃₋₂₉-17 Da from human serum incubations was

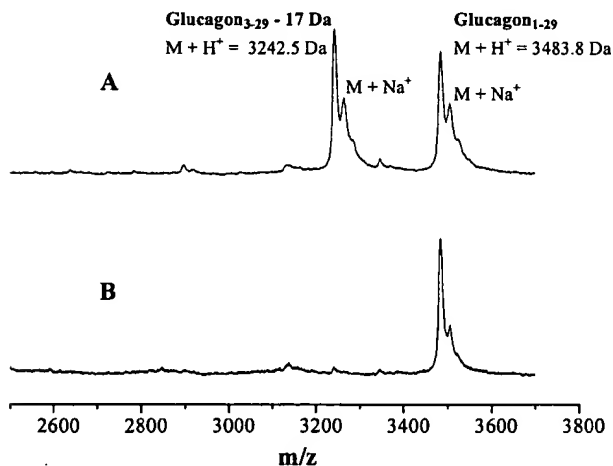


Fig. 3. Degradation of glucagon by 20% human serum monitored by MALDI-TOF mass spectrometry in the absence (A) and presence (B) of the DP IV inhibitor, Ile-Thia. Refer to Section 2 for specific methods.

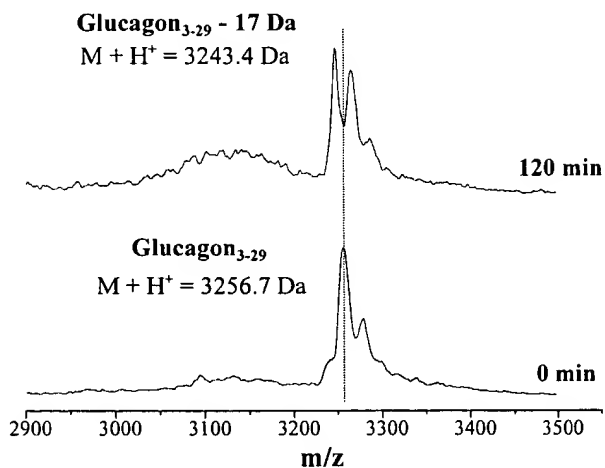


Fig. 4. Formation of a cyclized derivative of glucagon₃₋₂₉, measured by MALDI-TOF mass spectrometry. Glucagon₃₋₂₉ is converted by human serum to a peptide 17 Da smaller, the equivalent mass of an amide group.

indistinguishable from synthetic [pGlu³]glucagon₃₋₂₉ by MALDI-TOF MS, HPLC and capillary electrophoresis (data not shown).

Moreover, incubation of both peptides, glucagon₁₋₂₉ and glucagon₃₋₂₉, in 20% human serum and in the presence of 10 μ M of the highly specific, irreversible inhibitor of DP I,

N-(H-Gly-Phe-), *O*-benzoyl hydroxylamine, did not alter the observed glucagon degradation pattern (details to be published elsewhere).

3.2. Kinetic analysis of DP IV-mediated glucagon degradation

Kinetic characterization of the N-terminal degradation of glucagon_{1–29} to glucagon_{3–29} resulting from incubation of glucagon with purified porcine kidney DP IV was performed by capillary zone electrophoresis (Fig. 5). Molecular species were identified which corresponded to those observed by MALDI-TOF MS and time course studies were performed allowing calculation of enzyme kinetic parameters, K_m (Michaelis constant), k_{cat} (maximum velocity), and k_{cat}/K_m (specificity constant or second order rate constant). These results are summarized in Table 1 together with those for several other peptide substrates of DP IV taken from Mentlein et al. [5,32]. Notably, the

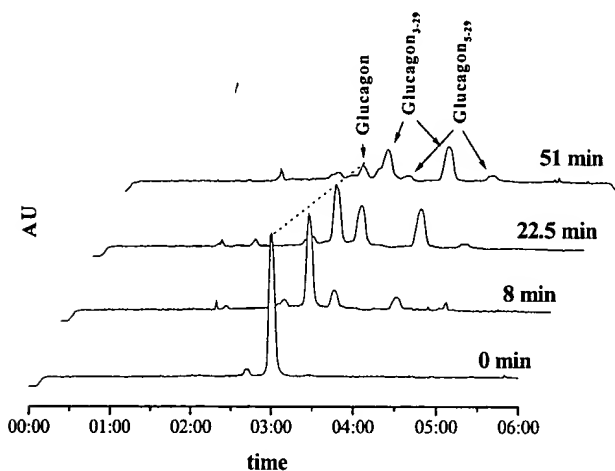


Fig. 5. Degradation of glucagon by purified porcine DP IV measured with capillary zone electrophoresis. Kinetic parameters of results can be found in Table 1. See Section 2 for specific methods. Clear sequential degradation to glucagon_{3–29} and glucagon_{5–29} is shown. Note that the latter fragments appear as double peaks, an effect likely due to the resolution of *cis/trans* isoforms of the truncated molecules. The same can be shown for purified synthetic glucagon_{3–29}.

Table 1
Kinetic parameters of DP IV-catalyzed peptide hydrolysis, determined by chromatographic methods

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{mM}^{-1} \text{s}^{-1}$)
Glucagon _{1–29} ^a	3.9	0.74	194
GIP ^b	34	7.6	220
GLP-1 _{7–36} ^b	4.5	1.9	430
PYY ^c	28	52	1900
NPY ^c	20	22	12 000

^a Parameters estimated by capillary zone electrophoresis (Materials and methods).

^b Data from Ref. [5] determined by HPLC.

^c Data from Ref. [32] determined by mass spectrometry.

enzymatic cleavage of the first dipeptide (His¹-Ser²) occurred at a more rapid rate than did the removal of the second (Gln³-Gly⁴).

3.3. Bioassay analysis of DP IV degradation

Degradation of glucagon in purified DP IV and rat plasma was investigated using a bioassay for the hyperglycemic effect of glucagon. Incubation of glucagon_{1–29} in purified DP IV or normal male Wistar rat plasma resulted in a significant loss of its hyperglycemic activity, when injected into either conscious or anesthetized Wistar rats (Figs. 6 and 7 respectively). Conversely, 50 μM Ile-Thia completely blocked the loss of bioactivity under the same conditions (Fig. 7). Comparison of the bioactivity of glucagon incubated in DP IV-negative or Fischer rat plasma shows that bioactivity is lost when incubated in Fischer plasma, but is only slightly reduced after incubation in DP IV-negative plasma relative to glucagon in saline (Fig. 6B). Bioassay of synthetic glucagon_{3–29}, [pGlu³]glucagon_{3–29}, and glucagon_{5–29} indicated that these peptides had negligible hyperglycemic activity at ten-fold higher doses than native glucagon (Fig. 6C).

4. Discussion

As discussed in the Introduction, there has been considerable controversy in the literature regarding the specific tissues and enzymes responsible for the degradation of glucagon. The current study specifically examined the possible involvement of the ubiquitous enzyme, dipeptidyl peptidase IV, in the metabolism of glucagon. Assessment of glucagon degradation products by capillary zone electrophoresis and MALDI-TOF spectrometry has identified the specific molecular species produced from glucagon metabolism both in human serum and by purified porcine DP IV. The data provide strong evidence that DP IV is a primary candidate for enzymatic degradation of glucagon.

Previous reports on degradation of glucagon by DP I were not confirmed. Similar to DP IV, DP I removes amino-terminal dipeptides, however, DP I is a promiscuous enzyme with a much broader substrate specificity [15–17,33]. In contrast to DP IV, DP I acts intracellularly, on the internal surface of lysosomal membranes, while DP IV is present as a membrane-bound ectoenzyme and a freely circulating enzyme [17,24,25]. Inhibition of DP I activity by specific inhibitors had no effect on the degradation of glucagon in human serum, indicating that DP I was not the enzyme responsible for glucagon degradation observed in serum. In contrast, inhibition of DP IV by Ile-Thia completely blocked serum mediated glucagon degradation (Fig. 2). Further support for the role of DP IV in glucagon metabolism was found using purified pig kidney DP IV, which gave rise to the expected N-terminally truncated peptide, glucagon_{3–29} in addition to an N-terminally

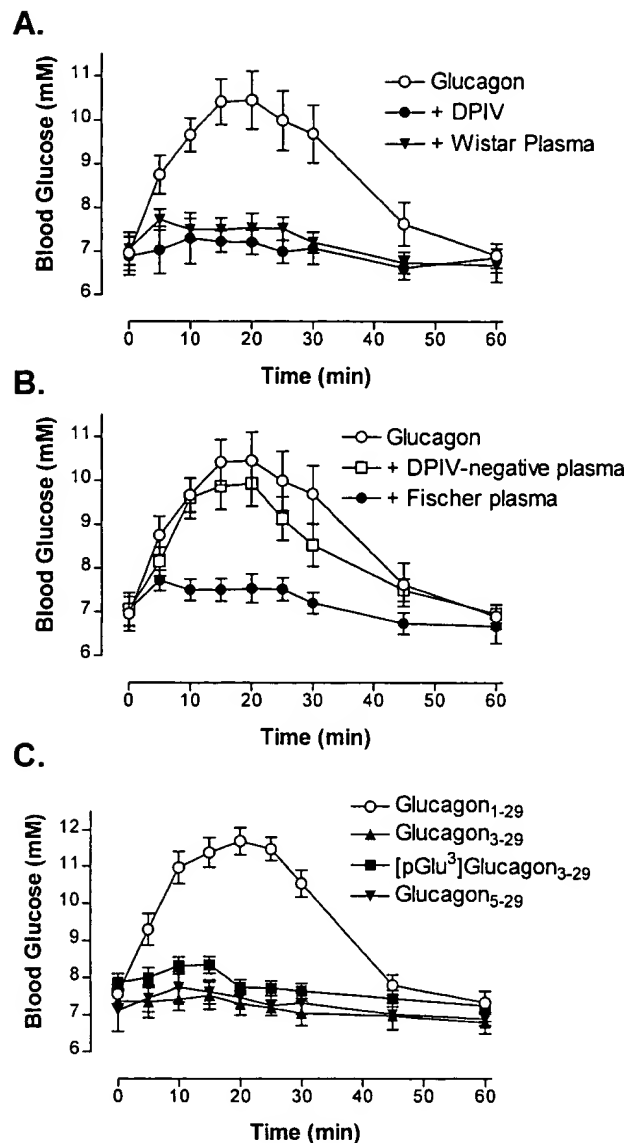


Fig. 6. Degradation and bioactivity of glucagon and N-terminally truncated analogues in vivo. (A and B) Glucagon (7.1 nmol/kg) was incubated in 1 ml saline, 0.31 mU purified DP IV, or rat plasma (37°C, 3.25 h) prior to subcutaneous injection into unrestrained, conscious male Wistar rats. (C) Synthetic glucagon analogues (71 nmol/kg) corresponding to DP IV degradation products were similarly bioassayed. Blood glucose was measured using a SureStep blood glucose analyzer. N = 6 for each group.

truncated product, glucagon₅₋₂₉, the result of a second N-terminal dipeptide cleavage step (Fig. 1). During preparation of this manuscript, another research group reported a similar sequential degradation of macrophage-derived chemokine (MDC) by DP IV [34]. Together with this report we have now shown that all known members of the GRF-superfamily of polypeptides are substrates for DP IV hydrolysis (data to be published elsewhere).

When examining degradation of glucagon by purified DP IV, the sequential loss of dipeptides resulted in the

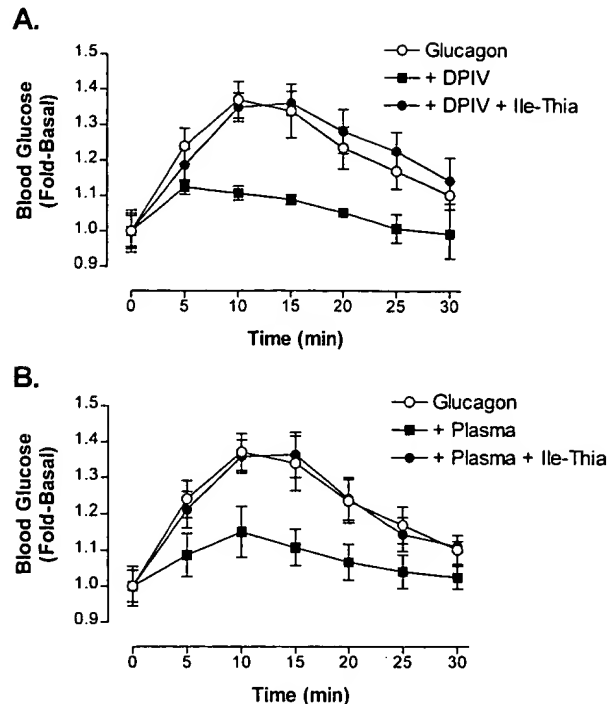


Fig. 7. Inhibition of DP IV and plasma degradation of glucagon by Ile-Thia monitored by bioassay. (A) Glucagon (7.1 nmol/kg) was incubated in saline with purified DP IV in the presence or absence of Ile-Thia (37°C, 3.25 h), prior to intravenous injection into anesthetized male Wistar rats. (B) Glucagon was similarly incubated in normal male Wistar rat serum with or without Ile-Thia, prior to IV injection. N = 4 for each group.

expected molecular weights for the truncated N-terminal fragments glucagon₃₋₂₉ and glucagon₅₋₂₉ (Fig. 1). However, incubation of glucagon₁₋₂₉ in serum consistently gave rise to a peptide product 17 Da less than the expected weight for glucagon₃₋₂₉: glucagon₃₋₂₉ - 17 Da (Figs. 2 and 3). Incubation of glucagon₃₋₂₉ in human serum also showed a rapid conversion to glucagon₃₋₂₉ - 17 Da (Fig. 4). Hence it appears that in serum, DP IV first cleaves the N-terminal dipeptide from glucagon, and a serum enzyme then catalyzes the cyclization of the amino terminal glutamine residue (Gln³), resulting in the loss of an NH₃ group (17 Da). Transglutaminases are well documented serum enzymes which cause the formation of new γ -carbox-amide bonds between the γ -carboxyl groups of glutamine and primary amines [35]. No intermediate glucagon₃₋₂₉ was observed in human serum, therefore the DP IV-catalyzed N-terminal cleavage reaction appears to be rate determining. Furthermore, as glucagon₅₋₂₉ was not observed, it appears that the cyclization step occurs much more rapidly than the second DP IV-mediated truncation step (cleavage of glucagon₃₋₂₉ to glucagon₅₋₂₉). In order to render the peptide resistant to further degradation by DP IV, the cyclization event must involve either the α -amine or γ -carbox-amide of Gln³. Products with the correct molecular mass could include pyroglutamyl-glucagon₃₋₂₉

([pGlu³]glucagon_{3–29}), cyclo^{1–12}-glucagon_{3–29}, cyclo^{1–20}-glucagon_{3–29} or cyclo^{1–24}-glucagon_{3–29}. When used as a standard for HPLC, MALDI–TOF MS, and CE, synthetic [pGlu³]glucagon_{3–29} could not be discriminated from the serum-catalyzed glucagon_{3–29} – 17 Da. Hence, it is likely that this is the product formed in serum, however, the possibility of other cyclic derivatives formed in vivo needs further investigation. The characteristics of this cyclization are consistent with the activity of the serum enzyme, glutaminyl cyclase [36].

In vitro enzymology studies identify DP IV as the primary enzyme involved in the metabolism of glucagon in the circulation. Further studies were carried out using a bioassay in rats to substantiate in vitro results and determine the in vivo consequences of N-terminal truncation and cyclization. Incubation of glucagon in normal rat plasma or purified porcine DP IV resulted in a complete loss of the hyperglycemic response to glucagon in vivo (Fig. 6A). Consistent with the in vitro assays, use of Ile-Thia prevented the loss of bioactivity of glucagon incubated in serum or DP IV (Fig. 7). Further evidence supporting DP IV-mediated metabolism of glucagon was provided by incubation of glucagon in DP IV-negative rat plasma. As expected, glucagon incubated in DP IV-negative plasma retained bioactivity, in contrast to control Fischer rat plasma (Fig. 6B). Concurrently with this study, the binding and activation characteristics of the truncated glucagon fragments were assessed in vitro using cells transfected with the human glucagon receptor [33]. All three peptides (glucagon_{3–29}, [pGlu³]glucagon_{3–29}, and glucagon_{5–29}) showed weak agonist activity with five- to 18-fold lower binding affinities than the native glucagon molecule. The current data are in agreement with these findings.

In addressing the physiological relevance of DP IV-mediated glucagon degradation in vivo, it is important to consider several points: first, the majority of the DP IV activity in the circulation is membrane bound, endothelial DP IV (at least one order of magnitude higher than plasma DP IV) [37], and therefore, the majority of the conversion of glucagon to its truncated form, in vivo, likely occurs at the blood vessel wall rather than in the plasma matrix. Among peptide substrates, glucagon exhibits the highest affinity for DP IV tested so far [25,38], in addition to a second-order rate constant on par with those of the incretins GIP and GLP-1 (Table 1). Taken together with the fact that the circulating concentration ranges of the three hormones are similar and the physiological impact of DP IV activity on incretin action, it is likely that DP IV plays a significant role in the clearance of active glucagon from the circulation. This is supported by our parallel characterization of D-Ser²-glucagon, a synthetic DP IV-resistant glucagon analog with receptor binding and activation characteristics equivalent to those of native glucagon. Using the same bioassay as outlined above, D-Ser²-glucagon was shown to elicit a prolonged and increased

hyperglycemic response when compared to native glucagon [30].

Comparison of the second-order rate constants of the three glucoregulatory peptides discussed so far, with those of previously characterized bioactive peptide DP IV substrates, now becomes very fitting. That is to say, glucagon, GIP, and GLP-1 ($k_{cat}/K_m \sim 200\text{--}400 \text{ mM}^{-1} \text{ s}^{-1}$), which must move through the circulation to their target tissues are afforded a relative DP IV-resistance over, for example, the locally acting neuropeptide DP IV-substrates NPY and PYY, which are degraded much more rapidly ($k_{cat}/K_m = 2000$ and $12\,000 \text{ mM}^{-1} \text{ s}^{-1}$, respectively; Table 1; [32]). Potential overstimulation of local paracrine pathways (e.g. glucagon stimulation of insulin secretion) by the more stable peptide hormones is likely avoided by co-release of DP IV and localization of DP IV to the target tissues. A recent finding of Grondin and colleagues, the localization of DP IV to the pancreatic α -cell secretory granule [39] gives support to this idea, as well as giving rise to the possibility of local effects of the truncated peptide fragments (dipeptides and N-terminally truncated).

As the glucagon degradation products (glucagon_{3–29} and [pGlu³]glucagon_{3–29}, or other possible cyclized derivatives) are found in human serum, measurements of glucagon immunoreactivity by 'side-viewing' or C-terminally directed antibodies [40] would likely cross react with these peptides. Studies using antibodies for the measurement of glucagon may therefore be misleading and this is likely the reason that glucagon was previously reported to be stable in plasma [14,41]. Frandsen et al. found that glucagon_{5–29} cross-reacted with at least two antisera tested (K5563 and K4023) [42]. Many such antibodies probably also cross-react with glucagon_{3–29} and [pGlu³]glucagon_{3–29} (or other cyclized derivatives). GLP-1_{7–36} is degraded to GLP-1_{9–36} by DP IV [4]. An N-terminal antibody to GLP-1_{9–36} exists, allowing determination of circulating levels of intact GLP-1, determined by a subtractive method. The circulating concentration of GLP-1_{9–36} was found to be approximately ten-times higher than the full length hormone [43]. Work is underway to develop assay techniques (both ELISA and radioimmunoassay) specific for the active N-terminally intact, as well as the cyclized and truncated, glucagon forms. Such assays, in addition to studies with larger animal models, are necessary for definitive characterization of the conversion of glucagon_{1–29} to [pGlu³]glucagon_{3–29} in vivo.

Considerable attention has been given to the development of DP IV resistant GLP-1 [44–47] and GIP [48,49] analogues, or inhibition of DP IV [8,10,12] to improve glucose tolerance in type 2 diabetic patients. Generation of synthetic incretin analogs with increased resistance to DP IV has led to peptides with increased circulating half-lives and enhanced antidiabetic effects owing to prolonged insulinotropic activity [44,47,49]. Similarly, inhibition of DP IV with the specific inhibitors, Ile-Thia and valylpyrrolidine, improved glucose tolerance in animal models,

likely through enhancing the activity of endogenous incretins [8,10,12]. Given that DP IV is also responsible for degrading glucagon, the improved glucose tolerance seems counter-intuitive, as inhibition of DP IV would also enhance the activity of endogenous glucagon. However, postprandially, both the stimulation, and thus the rate of endogenous glucagon secretion, are at a minimum, while those of the incretins are a maximum. Thus any enhancement of glucagon activity is likely masked by a much greater relative enhancement of incretin activity. Also, remaining intact negative feedback loops for glucagon continue to be active (in particular the glucagonostatic action of GLP-1 is likely enhanced), while the secretion stimulus for the incretins continues. It follows then, that when considering the design of DP IV inhibitors as antidiabetic agents, rapidly reversible inhibitors that are quickly cleared from the circulation are most desirable. Considering the data presented so far, it is likely that the physiological consequences of DP IV inhibition shift in tandem with this transition in regulatory control, from an insulin enhancing effect (incretin stabilizing) to a glucagon enhancing one (glucagon stabilizing) due to the relative changes in concentration of these circulating hormones in the postprandial state. When taken together with the fact that approximately two thirds of the 24 h cycle is spent in the fasting state, primarily under the control of glucagon, it is obvious that slow-binding, irreversible or stable inhibitors which sustain DP IV inhibition into the fasting state, would likely become counterproductive as antidiabetic drugs.

In summary, this study of glucagon metabolism has shown that DP IV is a primary enzyme involved in glucagon degradation. Purified porcine kidney DP IV was shown to sequentially degrade glucagon_{1–29} to glucagon_{3–29} and then glucagon_{5–29}. In human serum, cyclization of the truncated N-terminus prevented DP IV-mediated hydrolysis beyond glucagon_{3–29}. In vitro and in vivo, the amino-terminal truncation of glucagon was specifically blocked by the DP IV inhibitor Ile-Thia. Bioassay of synthetic fragments corresponding to DP IV degradation products revealed that truncated fragments possessed no hyperglycemic activity in vivo, leaving the physiological role of these peptides unknown. These results comprise a major step in the characterization of glucagon metabolism and thus contribute towards our understanding of diseases involving abnormal glucose counter-regulation.

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